

Cell cycle analysis and chromosomal localization of human Plk1, a putative homologue of the mitotic kinases *Drosophila polo* and *Saccharomyces cerevisiae* Cdc5

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SUMMARY

polo and *CDC5* are two genes required for passage through mitosis in *Drosophila melanogaster* and *Saccharomyces cerevisiae*, respectively. Both genes encode structurally related protein kinases that have been implicated in regulating the function of the mitotic spindle. Here, we report the characterization of a human protein kinase that displays extensive sequence similarity to *Drosophila polo* and *S. cerevisiae* Cdc5; we refer to this kinase as Plk1 (for polo-like kinase 1). The largest open reading frame of the *Plk1* cDNA encodes a protein of 68,254 daltons, and a protein of this size is detected by immunoblotting of HeLa cell extracts with monoclonal antibodies raised against the C-terminal part of Plk1 expressed in *Escherichia coli*. Northern blot analysis of RNA isolated from human cells

and mouse tissues shows that a single *Plk1* mRNA of 2.3 kb is highly expressed in tissues with a high mitotic index, consistent with a possible function of Plk1 in cell proliferation. The *Plk1* gene maps to position p12 on chromosome 16, a locus for which no associations with neoplastic malignancies are known. The Plk1 protein levels and its distribution change during the cell cycle, in a manner consistent with a role of Plk1 in mitosis. Thus, like *Drosophila polo* and *S. cerevisiae* Cdc5, human Plk1 is likely to function in cell cycle progression.

Key words: cell cycle, protein kinase, mitosis, polo, Cdc5, mitotic spindle

INTRODUCTION

Accurate segregation of genetic material during meiosis and mitosis is essential for cell viability, and hence is an important event in the cell cycle. The segregation of sister chromatids during mitosis, or of homologous chromosomes during meiosis, requires the assembly and proper function of a microtubule spindle (reviewed by McIntosh and Koonce, 1989). Multiple lines of evidence suggest that protein phosphorylation plays a key role in controlling the assembly and dynamics of the mitotic spindle, and in chromosome segregation (Hyman and Mitchison, 1991; Karsenti, 1991; Verde et al. 1991; Buendia et al., 1992; Kalt and Schliwa, 1993), but the identity of most of the protein kinases and phosphatases involved in these processes, as well as their corresponding substrates, is not known.

Genetic and cytological evidence implicates two structurally related protein kinases, polo of *Drosophila melanogaster* (Sunkel and Glover, 1988; Llamazares et al., 1991), and Cdc5 of *Saccharomyces cerevisiae* (Hartwell et al., 1973; Byers and

Goetsch, 1974; Schild and Byers, 1980; Sharon and Simchem, 1990), in the control of spindle function and chromosome disjunction. The protein kinase polo was identified by analysis of *Drosophila* mutants that arrest in late mitosis (for review see Glover, 1991). Whereas one allele of *polo* caused lethality at the larval stage, another allele elicited a broad range of mitotic phenotypes, including cells with abnormal spindle poles, aberrant chromosome distributions, and monopolar or highly branched bipolar spindles with overcondensed chromosomes (Sunkel and Glover, 1988; Llamazares et al., 1991).

A recent study has revealed substantial sequence similarity between the *Drosophila polo* kinase and the product of a cell division cycle mutant gene, *CDC5*, of *S. cerevisiae* (Kitada et al., 1993). In line with the results obtained with *Drosophila polo* mutants, *CDC5* mutants of *S. cerevisiae* also display mitotic and meiotic arrest in late nuclear division (Hartwell et al., 1973; Byers and Goetsch, 1974), with frequent anomalies in the microtubule spindle (Schild and Byers, 1980) and failure to undergo reductional segregation of chromosomes in meiosis I (Sharon and Simchem, 1990). Taken together, these obser-

variations indicate that the protein kinases encoded by *polo* and *CDC5* may represent functional homologues in *Drosophila* and *S. cerevisiae*, respectively. Both kinases are clearly required for passage through later stages of mitosis. These genetic data are supported by the recent demonstration that the kinase activity of *Drosophila* polo peaks at the anaphase to telophase transition (Fenton and Glover, 1993).

Here we describe a human protein kinase that shows a high degree of sequence similarity to *Drosophila* polo, and hence is called Plk1 (for polo like kinase 1). This kinase is also closely related to Cdc5 of *S. cerevisiae*, and the recently described murine protein kinases, Snk (Simmons et al., 1992) and Plk (Clay et al., 1993). Sequence conservation between these kinases is not confined to the N-terminally located catalytic domains but extends throughout the entire proteins. In particular, the C-terminal end domains of these proteins contain highly conserved sequence motifs that may define a novel subfamily of protein kinases. We also present evidence that human Plk1 may have a role in mitosis in mammalian cells. Plk1 mRNA levels are highest in tissues that contain dividing cells and Plk1 protein levels fluctuate during the cell cycle, being highest in mitosis.

MATERIALS AND METHODS

Cloning and sequencing of human *Plk1*

cDNA fragments spanning parts of the catalytic domains of human protein kinases were amplified by PCR, as described by Schultz and Nigg (1993). A 144 bp fragment (named HsPK28) showing 88% nucleotide identity to *Drosophila polo* was used to probe a λ gt 10 library prepared from a human nasopharyngeal carcinoma (Hitt et al., 1989). Approximately 500,000 plaques were screened by plaque hybridization (Sambrook et al., 1989), and 17 phage showing strong hybridization were purified. DNA was prepared using LambdaSorb (Promega Biotech, Madison, WI). One plasmid (referred to as Plk1-pGEM) with a 2143 bp insert contained the entire coding sequence for a protein kinase closely related to *Drosophila* polo. This insert was sequenced on both strands by the method of Chen and Seeburg (1985), using the Sequenase kit (United States Biochemicals). Sequence comparisons were made using the University of Wisconsin GCG package and PC/GENE software.

Expression of a human Plk1 fragment in *E. coli*, production of monoclonal antibodies and immunochemical techniques

The plasmid Plk1-pQE9 was prepared by subcloning a 880 bp *Bam*HI fragment of *Plk1* into the *Bam*HI site of the vector pQE9 (Diagen, Dusseldorf, Germany). Induction of transformed bacteria yielded the expression of a 25 kDa fusion protein, termed C-termPlk1, that contained amino acids 402-603 of Plk1, preceded by the polyhistidine sequence MRGSHHHHHHT. C-termPlk1 was purified by nickel column chromatography, exactly as described by the manufacturers of the Qiaexpress kit (Diagen). Balb/c mice were immunized by repeated subcutaneous injections of 50 μ g of C-termPlk1. Fusion of spleen cells to NS-2 mouse myeloma cells, dot blot assays for supernatant screening, hybridoma cloning and mAb isotyping were performed as described previously (Lukas et al., 1992). The mAb PL6 used predominantly in this study is an IgG₁. For immunoblot analysis PL6 ascites fluid, affinity-purified rabbit anti-cyclin A antibody (Maridor et al., 1993) and affinity-purified rabbit anti-p34^{cdc2} (R8; Krek and Nigg, 1991b) were diluted in phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.2) containing 1% nonfat milk and 0.2% Tween-20.

They were used at final concentrations of 4 μ g/ml, 1 μ g/ml and 2 μ g/ml, respectively. Immunoreactive bands were visualized using appropriate anti-rabbit or anti-mouse secondary antibodies conjugated to alkaline phosphatase (Promega). For indirect immunofluorescence microscopy, HeLa cells were cultured on glass coverslips and then treated either with 3% paraformaldehyde, 2% sucrose in PBS (5 minutes, room temperature), followed by 0.5% Triton X-100 in PBS (5 minutes, 4°C), or with methanol (5 minutes, -20°C), followed by acetone (30 seconds, -20°C). For stabilization of microtubules, formaldehyde-fixed cells were permeabilized for 5 minutes at room temperature with 0.5% Triton X-100 in 20 mM HEPES, pH 7.4, 3 mM MgCl₂, 50 mM NaCl, 300 mM sucrose and 0.02% sodium azide, followed by a 5 minute incubation in methanol (-20°C). Primary antibodies were added at room temperature for 30 minutes. mAb PL6 ascites fluid was diluted 1:500 in PBS containing 3% BSA, 0.02% sodium azide, whereas the anti-myc-tag mAb 9E10 (Evan et al., 1985) was used as hybridoma supernatant and rat anti-tubulin mAb YOL1/34 (Serotec) was diluted 1:1 in PBS. Secondary antibodies were Cy3 anti-mouse IgG (Jackson ImmunoResearch Laboratories) and FITC-conjugated goat anti-rat IgG (Cappel Laboratories). Double staining was performed by addition of antibodies in the following order: anti-Plk1 mAb PL6, anti-mouse secondary antibody, anti-tubulin YOL1/34, anti-rat secondary antibody. DNA was detected by addition of Hoechst dye 33258 at a concentration of 5 μ g/ml to the secondary antibodies.

Transient expression of Plk1 in HeLa cells

To express a full-length Plk1 protein, the 1998 bp *Hind*III-*Apal* fragment of Plk1-pGEM was introduced into the pRC/CMV vector (Invitrogen Corporation, San Diego), to create Plk1-CMV. An N-terminally myc-tagged Plk1-CMV was also constructed. In the resulting mycplk1 protein, the N terminus of Plk1 is extended by the oligopeptide MEQKLISEEDLNMSNSCPGS (Evan et al., 1985). HeLa cells were transfected in 60 mm culture dishes, exactly as described by Krek and Nigg (1991a).

Northern analyses

Total RNA was isolated from tissues and cell lines according to Chomczynski and Sacchi (1987), and poly(A)⁺ mRNA was selected using oligo-dT (Sambrook et al., 1989). Aliquots (5 μ g) of each mRNA were denatured with glyoxal, analyzed by electrophoresis on a 1% agarose gel and transferred to nitrocellulose by filters. Filters were prehybridized for 4 hours in 50% formamide, 4 \times SSC, 5 \times Denhardt's, 100 μ g/ml denatured salmon sperm DNA, 0.2% SDS, 0.1% sodium pyrophosphate, and hybridized for 18 hours at 50°C in the same solution with a ³²P-labeled 2143 bp *Eco*RI fragment of the Plk1-pGEM plasmid. Filters were washed to a final stringency of 2 \times SSC, 0.5% SDS at 50°C before exposure to X-ray film.

Chromosomal localization of *Plk1*

The chromosomal mapping position of the *Plk1* cDNA was determined by fluorescence in situ hybridization. Metaphase chromosomes were prepared according to standard procedures (Verma and Babu, 1989). Mapping of *Plk1* and digital image analysis were carried out as described previously (Ried et al., 1992a,b). A total of 15 randomly selected metaphases were evaluated: eight revealed hybridization signals on both chromatids of the homologous chromosomes; five metaphases showed signals only on one chromatid or only one homologue; two metaphases did not display any signal; and signals on different mapping positions were not observed.

Cell cycle analysis by centrifugal elutriation and nocodazole arrest-release

HeLa suspension cells (1 \times 10¹⁰) were cultured in S-MEM spinner culture medium (Gibco) supplemented with 5% fetal calf serum and penicillin-streptomycin (100 i.u./ml and 100 μ g/ml, respectively) and then sorted by the method of centrifugal elutriation as described

canonical consensus sequence GxGxxG (Hanks and Quinn, 1991). Furthermore, a highly conserved valine residue normally found two residues downstream of the GxGxxG motif is replaced by cysteine (Fig. 2A). Across the 252 amino acid protein kinase catalytic domain, Plk1 shares 65% sequence identity with polo, 53% identity with Snk, and 50% identity with Cdc5. Over the entire protein, Plk1 displays 52%, 38% and 31% identity to polo, Snk and Cdc5, respectively.

The region immediately C-terminal to the catalytic domain (corresponding to residues 305 to 410 in Plk1) shows very little sequence conservation between these four kinases, and in part accounts for their difference in size. However, as shown in Fig. 2B, there is substantial sequence conservation in the C-terminal end domains, supporting their classification into a novel kinase subfamily. Sequence identities are scattered throughout the C-terminal end domains, with the most strikingly conserved motif, S/TKWVDYSxKxGxxYQL, beginning at residue 412 of Plk1; this sequence may constitute a hallmark of polo-like kinases.

Immunological identification of Plk1 protein in HeLa cells

We have confirmed that the Plk1 sequence shown in Fig. 1 represents the full-length protein using monoclonal antibodies (mAbs) raised against the C-terminal end domain. Immunoblotting of total HeLa cell extracts with mAb PL6 revealed a major band that migrated with an apparent molecular mass of 68 kDa (Fig. 3, lane 1); occasionally, a fainter band at 110 kDa was also visible (but difficult to

reproduce photographically). Whereas the relationship between this minor 110 kDa band and Plk1 is not known, the 68 kDa protein comigrated with a strongly immunoreactive band observed after transfection of HeLa cells with the *Plk1* cDNA (Fig. 3, lane 2), indicating that this band represents endogenous Plk1. When HeLa cells were transfected with a plasmid encoding a myc-tagged Plk1 construct, the Plk1 protein displayed a retarded electrophoretic mobility, as expected in view of its increased size (Fig. 3, lane 3). These results demonstrate that mAb PL6 recognizes Plk1, and they indicate that the *Plk1* cDNA described here encodes the full-length protein.

Expression pattern and chromosomal localization of *Plk1* gene

In northern blot analyses carried out on total RNA isolated from HeLa, 3229 fibroblast and M14 melanoma cell lines, a single band migrating at 2.3 kb was detected (Fig. 4A). Thus, the *Plk1* message is approximately 150 bp larger than the 2137 bp cDNA described here. To examine tissue-specific expression of *Plk1*, we analyzed a number of different mouse tissues (Fig. 4B). Northern blot analysis revealed high expression of the 2.3 kb *Plk1* mRNA in testis, spleen and thymus of adult (2-4 month old) mice, as well as in mRNA preparations of total 13-day-old mouse embryos. Little or no *Plk1* message was detected in pancreas, heart, skin, brain and kidney, and moderate levels were seen in lung, placenta, ovary and intestine. This survey suggests that high levels of *Plk1* expression correlate with a high proliferative index of a tissue.

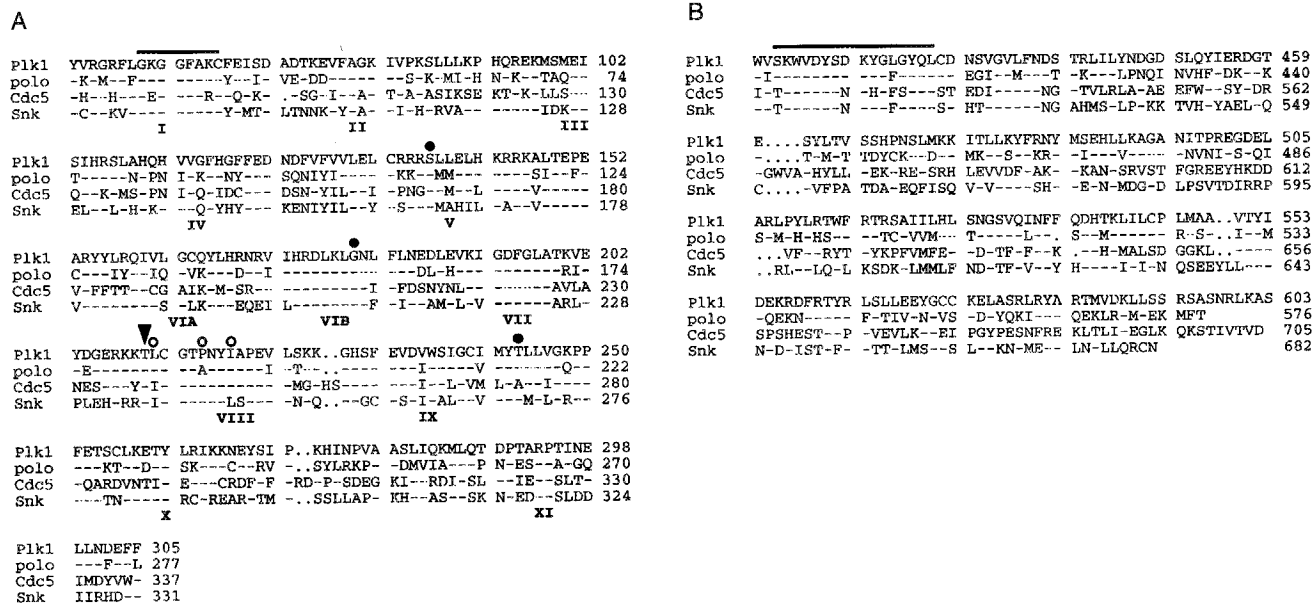


Fig. 2. Amino acid sequence identities between members of the polo-like kinase subfamily. (A) Sequence conservation across the catalytic domains of human Plk1, *Drosophila* polo, *S. cerevisiae* Cdc5, and murine Snk. The amino acid sequence of Plk1 is shown using the single-letter code. The sequences of the other kinases are indicated only at positions where they diverge from Plk1; identical residues are indicated by dashes. Points mark the positions where gaps were introduced to optimize alignments. A continuous line indicates the position of a conserved GxGxxAxG instead of the typical GxGxxGxV motif, the arrowhead points to a potential phosphorylation site, and filled and open circles mark residues that are implicated in making contact with substrate determinants, i.e. residues flanking phosphoacceptor serines/threonines on the N- and C-terminal side, respectively (Knighton et al., 1991; Bossemeyer et al. 1993). Subdomains are numbered according to Hanks et al. (1988). (B) Sequence alignment of C-terminal end domains of polo-like kinases. Sequences are represented as described above. The continuous line marks a strikingly conserved motif (S/TKWVDYSxKxGxxYQL), which may represent a characteristic feature of polo-like kinases.

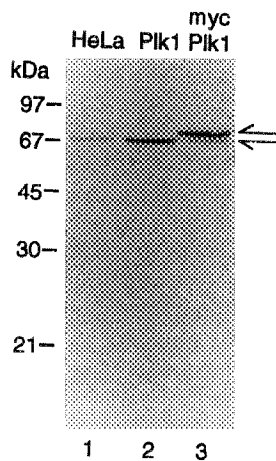


Fig. 3. Identification of endogenous and overexpressed Plk1 in HeLa cell extracts. Total cell lysates were prepared from exponentially growing HeLa cells (lane 1), and HeLa cells that had been transfected with cDNAs encoding Plk1 (lane 2) or mycplk1 (lane 3). Extracts were analyzed by immunoblotting, using the anti-Plk1 mAb PL6, and secondary antibodies coupled to alkaline phosphatase (Promega). Equal loading was confirmed by Coomassie Blue staining (not shown). Note the exact comigration of the endogenous protein detected in lane 1 with the overexpressed Plk1 protein in lane 2 (lower arrow). The decreased mobility of the mycplk1 protein detected in lane 3 (upper arrow) is consistent with the presence of the 20 amino acid myc-tag at the N terminus of Plk1. The positions of molecular mass markers are indicated in kDa.

In view of this correlation, it was of interest to determine the chromosomal location of the *Plk1* gene relative to loci that are implicated in human cancers. By the method of fluorescence in situ hybridization, a signal was observed on chromosome 16p11.2-13.1. The mapping position is schematically illustrated on a 400 band resolution ideogram in Fig. 5 (original data are available upon request). At present, this region does not contain any known chromosomal markers associated with

human cancers. Thus, further studies will be necessary to determine whether aberrant expression of Plk1 might contribute to neoplastic disease in humans.

Subcellular localization of Plk1 in HeLa cells

The subcellular localization of Plk1 was examined in exponentially growing HeLa cells using mAb PL6 for indirect immunofluorescence microscopy. The strongest staining was observed in cells that were in mitosis whereas interphase cells showed a signal of variable intensity (Fig. 6A). In most cells a diffuse fluorescence was seen in both cytoplasm and nucleus, but in cells that had just divided intense staining was seen in the region of the midbody (Fig. 6A, see arrow). This striking staining of the postmitotic bridge was observed using different antibodies and different procedures for fixation and permeabilization (e.g. paraformaldehyde/Triton X-100 or methanol/acetone) (data not shown). For further control of specificity, HeLa cells were transfected with a myc-epitope-tagged Plk1 cDNA (mycplk1), and then stained with mAb 9E10 directed against the myc epitope. As shown in Fig. 6C, the tagged Plk1 protein also localized to postmitotic bridges; this staining was independent of fixation methods and not seen in cells transfected with mycplk1 cDNA in the antisense orientation. Close examination of postmitotic bridges revealed that the Plk1 staining was concentrated near the midbody, in contrast to tubulin staining, which extended further into the cytoplasmic bridge (Fig. 6E and F). We conclude from these results that Plk1 is found throughout the cell during interphase, but localizes to the postmitotic bridge in cells that have just divided.

Plk1 protein expression is cell cycle-dependent

To investigate whether Plk1 might play a role in cell cycle progression we analyzed its protein expression in HeLa cells that had been synchronized by centrifugal elutriation (Fig. 7). The cell cycle stage of each population was determined by FACS analysis (Fig. 7B). In Fig. 7A, the levels of Plk1 protein in each fraction are compared with those of cyclin A and p34^{cdc2}. Closely resembling the expression pattern of cyclin A, Plk1

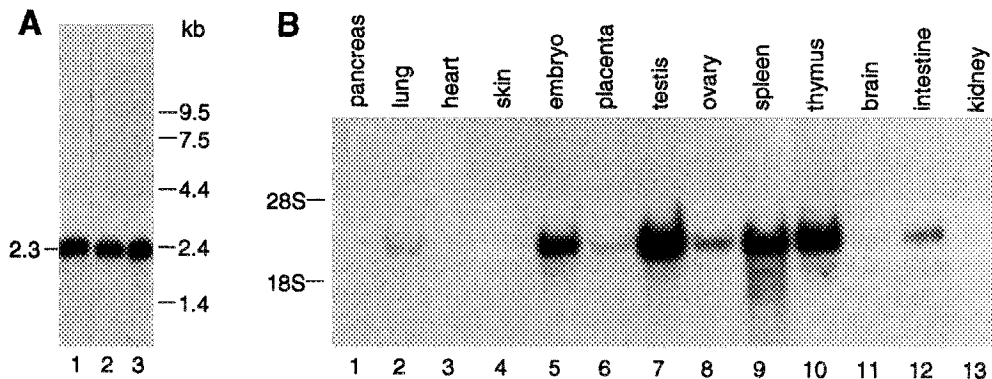
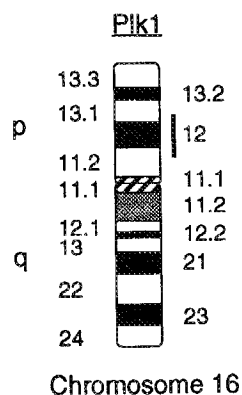


Fig. 4. Northern blot analysis of *Plk1* expression. (A) Total RNA was isolated from HeLa cells (lane 1), 3229 foreskin fibroblasts (lane 2), and M14 melanoma cells (lane 3). A 5 µg sample of RNA was electrophoresed in a 1% agarose gel, transferred to nitrocellulose, and probed with a ³²P-labeled 2143 bp *Plk1* fragment (2×10⁶ cpm/ml). Autoradiography was for 16 hours. Size standards (Gibco/BRL) are indicated in kb. (B) Poly(A)⁺ mRNA was isolated from the adult mouse tissues indicated, as well as from 13-day-old embryos. A 5 µg sample of RNA from each tissue was processed for northern blot analysis as described in A. Loading of equivalent amounts of RNA in each lane was confirmed by staining with ethidium bromide (not shown). The positions of ribosomal RNA species are shown on the left.

Fig. 5. The chromosomal location of *Plk1*. Shown is a schematic diagram that summarizes the location of *Plk1* on human chromosome 16. Data were obtained by hybridization of a 2143 bp *Plk1* cDNA probe to human metaphase chromosome spreads. The arms of the chromosome are marked on the left, and a vertical bar shows the *Plk1* site, which lies between positions 11.2 and 13.1.



protein was of very low abundance in populations that were enriched for G_1 phase cells (lanes 1-4). Levels increased gradually as cell populations approached S phase (lanes 5-9)

and reached maximal levels in populations with the highest proportion of cells in G_2/M phases (lanes 10-11). In contrast, $p34^{cdc2}$ levels were essentially constant in all samples, as expected (Draetta and Beach, 1988). Also, as cells approached G_2 phase, a $p34^{cdc2}$ protein with characteristically reduced electrophoretic mobility was seen, indicating tyrosine phosphorylation (Draetta et al., 1988). All three proteins were readily detected in exponentially growing cells (lane Ex).

To carry out a refined analysis of *Plk1* expression during mitosis, HeLa cells were arrested in prometaphase using nocodazole, and then released for various time intervals (Fig. 7C). Consistent with the results obtained by elutriation, levels of *Plk1* were high in cells arrested in M phase (Fig. 7C, lane 1), and 15 minutes (lane 2) and 30 minutes (lane 3) after release from the nocodazole arrest. They decreased as cells completed mitosis (lane 4; 1 hour time point) and re-entered G_1 phase (lane 5; 4 hour time point). These data suggest that *Plk1* levels drop some time during late mitosis/early G_1 (see Discussion),

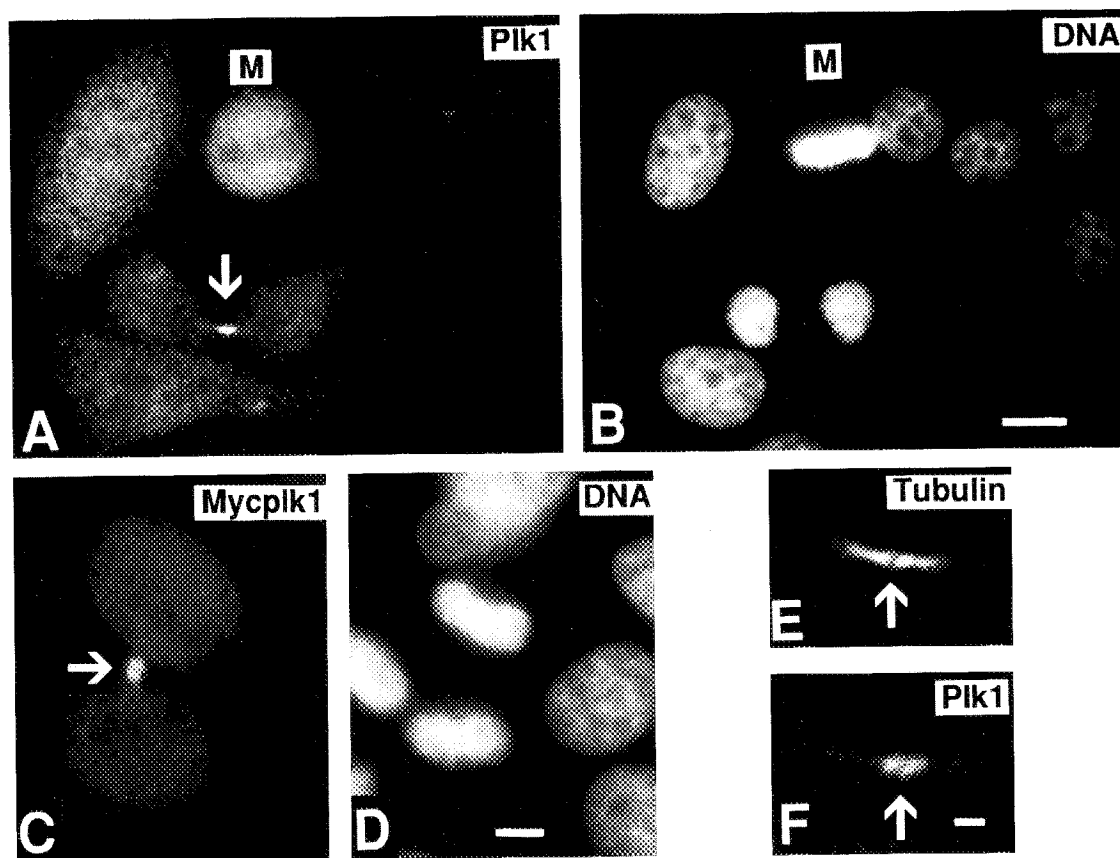


Fig. 6. Subcellular localization of *Plk1* in HeLa cells and mycplk1-transfected HeLa cells. (A) Exponentially growing HeLa cells were cultured on glass coverslips. They were treated for 5 minutes with 3% paraformaldehyde, 2% sucrose in PBS (room temperature), followed by 5 minutes in 0.5% Triton X-100 in 20 mM HEPES, pH 7.4, 3 mM $MgCl_2$, 50 mM NaCl, 300 mM sucrose and 0.02% sodium azide (room temperature), then for 5 minutes with methanol ($-20^\circ C$) and washed in PBS. Then, they were probed with mAb PL6, followed by Cy3 anti-mouse IgG secondary antibodies. Indirect immunofluorescence microscopy was carried out with a Zeiss Axiophot fluorescence microscope using a $\times 63$ objective. Note the striking variation in staining intensity amongst different cells, particularly the bright staining of the mitotic cell (labelled M). The arrow points to pronounced staining of a postmitotic bridge. (B) Hoechst 33258 DNA staining of the same cells shown in A. Note that the strongest staining cell of A corresponds to a cell in metaphase (M). Bar, 10 μm . (C) HeLa cells were transfected with mycplk1 cDNA. After 24 hours, cells were fixed and permeabilized as described above, stained with anti-myc-tag 9E10 mAb and Cy3 anti-mouse IgG secondary antibodies, and observed by indirect immunofluorescence microscopy. The arrow points to intense staining seen on either side of a midbody in a dividing cell. (D) Hoechst 33258 DNA staining of the same cells shown in C. Note that the 9E10 mAb stains only transfected cells, which is why many cells are not seen in C. Bar, 5 μm . (E and F) Comparison of *Plk1* staining (F) and tubulin staining (E) of a postmitotic bridge. Cells were fixed and permeabilized as described above, and tubulin was visualized using the anti-tubulin rat mAb YOL1/34. Bar, 2 μm .

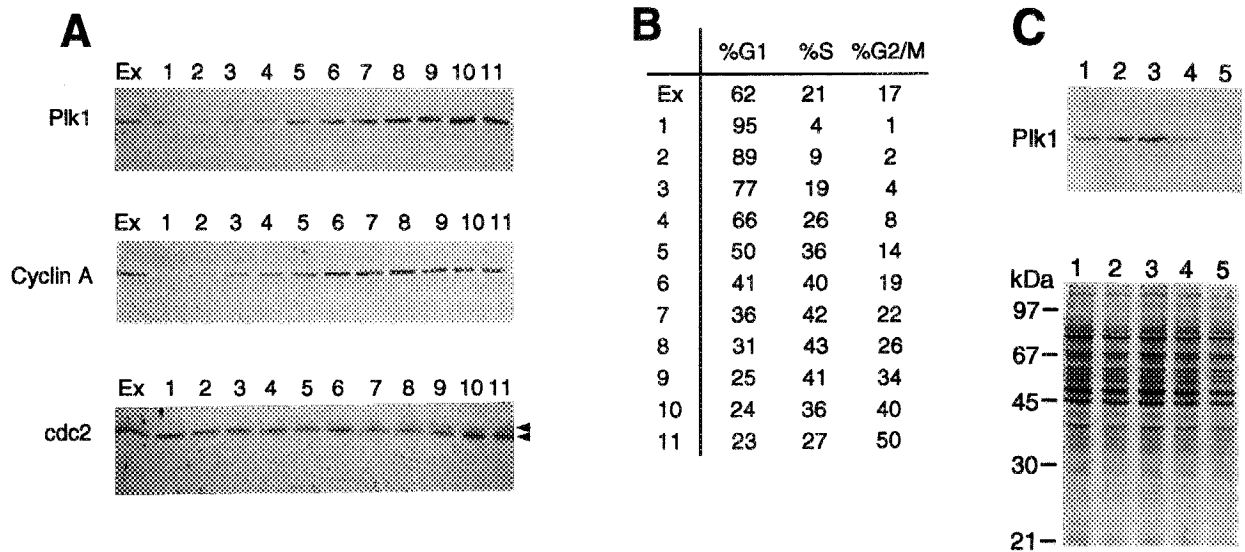


Fig. 7. Cell cycle-dependent expression of Plk1 protein. (A) Exponentially growing HeLa cells were fractionated by centrifugal elutriation. Each fraction was then analyzed by SDS-PAGE and immunoblotting. Equal sample loading was controlled by staining of gels in parallel with Coomassie Blue (not shown). The lane marked Ex represents an extract prepared from exponentially growing cells, whereas samples 1 through 11 represent cells at progressive stages of the cell cycle. All samples were probed with antibodies against Plk1, cyclin A or p34^{cdc2}, and immunoreactive proteins were visualized with alkaline phosphatase-conjugated secondary antibodies. The arrowheads mark the position of two p34^{cdc2} proteins differing in their phosphorylation state. Only the relevant portions of each immunoblot are shown. (B) One third of each elutriated cell sample was analyzed by FACS to determine the percentage of cells in G₁, S and G₂/M phases. The values listed for Ex and samples 1 through 11 correspond to the lanes shown in A. (C) Plk1 levels persist during mitosis. HeLa cells were cultured for 16 hours in the presence of nocodazole (100 ng/ml). Extracts were prepared for analysis by SDS-PAGE and immunoblotting with anti-Plk1 mAb PL6 at time 0 (lane 1), as well as 15 minutes (lane 2), 30 minutes (lane 3), 1 hour (lane 4) and 4 hours (lane 5) after removal of nocodazole. By 4 hours, the vast majority of cells had re-entered G₁ phase, as evidenced by their reattachment to the culture dish. Only the relevant part of the Plk1 immunoblot is shown; the lower panel shows Coomassie Blue staining of the samples, indicating that similar amounts of protein were loaded in each lane. The positions of molecular mass markers are shown on the left.

and they are entirely consistent with the results obtained by immunofluorescence microscopy (Fig. 6). Taken together, our results demonstrate that Plk1 protein expression is regulated during the cell cycle and peaks during mitosis.

DISCUSSION

Motivated by the evolutionary conservation observed for many key enzymes involved in cell cycle control, we have begun to isolate cDNAs for human protein kinases that are likely to be functional homologues of genes previously identified in organisms such as fungi and the fruit fly *Drosophila*. From a bank of 21 cDNAs encoding parts of kinase catalytic domains (Schultz and Nigg, 1993) we have here used a fragment showing a high degree of similarity to *Drosophila polo* to isolate a full-length human cDNA of a putative human homologue. Independently, an almost identical cDNA has been reported while this manuscript was in preparation (Lake and Jelinek, 1993) and the putative mouse homologue of Plk1 has also been isolated (Clay et al., 1993; Lake and Jelinek, 1993). The predicted amino acid sequence of Plk1 shows high sequence similarity to both *Drosophila polo* (Llamazares et al., 1991) and Cdc5 of the budding yeast *S. cerevisiae* (Kitada et al., 1993), and a substantial degree of similarity is revealed also to the murine kinase Snk (Simmons et al., 1992). Sequence similarity between these kinases is not confined to the N-ter-

minally located catalytic domain, but is observed also in parts of the C-terminal domain. In particular, all these kinases exhibit a 16 amino acid stretch that has not been described for any other protein in the data bases. It is not yet known if Plk1 is a functional homologue of polo or Cdc5, since our attempts to complement either *polo* or CDC5 mutant strains with human Plk1 have either failed or yielded inconclusive results.

By analogy to the crystal structure of the cAMP-dependent protein kinase, (PKA; for review see Taylor et al., 1993), a number of clues about the regulation and substrate specificity of Plk1 emerge from the analysis of its sequence. The structural features discussed below are also found among the four kinases listed in Fig. 2A, suggesting that certain aspects of regulation and substrate specificity may have been conserved between these kinases. With respect to possible regulation, it is interesting that the position of threonine 210 in Plk1 corresponds exactly to that of threonine 197 in PKA. In the case of PKA, phosphorylation of threonine 197 is thought to stabilize the kinase in a conformation appropriate for phosphotransfer (Knighton et al., 1991). The phosphate attached to threonine 197 of PKA displays a very low turnover (Shoji et al., 1979), but other protein kinases, including p34^{cdc2} (Gould et al., 1991; Krek and Nigg, 1992), are known to be regulated via phosphorylation at a corresponding site. Thus, it will be interesting to determine whether the activity of Plk1 requires phosphorylation of threonine 210.

So far, we have been unable to detect kinase activity asso-

ciated with immunoprecipitated Plk1 and thus have no direct information on its substrate specificity. However, it is noteworthy that three glutamic acids implicated in binding of positively charged substrate peptides by PKA (residues 127, 170 and 230; Knighton et al., 1991; Bossemeyer et al., 1993) are replaced in Plk1 by three non-charged amino acids (serine 137, glycine 180 and threonine 243; Fig. 2A, filled circles). This suggests that the substrates of Plk1 are unlikely to contain positively charged determinants amino-proximal (positions P⁻³ and P⁻²) to the target serine or threonine residues. On the other hand, Plk1 shares with PKA a cluster of hydrophobic amino acids (leucine 211, proline 215 and isoleucine 218; Fig. 2A, open circles) that have been implicated in establishing contacts with a hydrophobic C-terminal flanking residue (P⁺¹) of PKA target sites (Knighton et al., 1991; Bossemeyer et al., 1993). On the basis of these considerations, we predict that determinants for substrate recognition by Plk1 are likely to be hydrophobic at the P⁺¹ position, and unlikely to be positively charged at the P⁻² and P⁻³ positions. Consistent with this interpretation, both polo and Cdc5 were recently reported to phosphorylate (negatively charged) casein but not (positively charged) histone H1 (Kitada et al., 1993; Fenton and Glover, 1993).

Northern blot analysis of Plk1 in exponentially growing human tissue culture cells revealed a single message that corresponds closely to the size of the *Plk1* cDNA. Examination of RNA isolated from mouse tissues showed highest expression of *Plk1* message in tissues with a high proliferative index. These data support similar results reported for murine *Plk1* (Clay et al., 1993) and *Drosophila polo* mRNAs (Llamazares et al., 1991), but Lake and Jelinek (1993) observed high expression of murine *Plk1* only in testis, with weak signals being detectable in several other tissues. We do not know the significance of this difference in results. We also note that the mRNA expression of the distantly related murine kinase *Snk* was reported to be low in exponentially growing cells, but inducible with treatment of cells with phorbol esters or serum (Simmons et al., 1992). Although *Snk* shares several structural features with the mitotic kinases polo and Cdc5, its pattern of expression suggests that it may act at a different stage of the cell cycle. In this context, it is interesting also that the *S. cerevisiae* kinase Cdc5 was initially identified because of its ability to rescue a DBF4 mutant that arrests at G₁/S; yet detailed analysis of CDC5 mutant phenotypes revealed that this gene product has primarily a mitotic function (Kitada et al., 1993). Taken together, these observations raise the possibility that different members of the polo-like kinase family may function at different times during the cell cycle, a phenomenon reminiscent of the cdc2/cdk kinase family (for review see Pines and Hunter, 1991). It will be interesting to elucidate what regulatory mechanisms determine the timing of activation of different polo-like kinases.

Most importantly, human Plk1 protein was shown here to display a striking cell cycle-dependent pattern of expression. Immunoblotting analysis of HeLa cells synchronized by elutriation revealed that Plk1 expression closely parallels that of cyclin A, at least during interphase of the cell cycle. However, whereas cyclin A is destroyed during early stages of mitosis (Pines and Hunter, 1990), Plk1 persists through mitosis. This is illustrated by the results of nocodazole arrest-release experiments, which show that Plk1 levels remain high for 15 and 30

minutes after release from a nocodazole block in prometaphase, but decrease to a very low level by 4 hours, when cells re-enter the next G₁ phase (Fig. 7C).

Immunofluorescence microscopy showed that Plk1 protein concentrates within postmitotic bridges of dividing cells, whereas it is distributed diffusely throughout interphase cells. We have not observed any evidence for an association of Plk1 with mitotically condensed chromatin, contrary to results reported for *Drosophila polo* (Llamazares et al., 1991), but this may reflect differences in the developmental stages of the analyzed cell types. The subcellular localizations of *S. cerevisiae* Cdc5 or murine Plk and Snk have not been reported. Accumulation of Plk1 to either side of the midbody of dividing HeLa cells was detected when visualizing either endogenous protein or an ectopically expressed tagged version of Plk1. The functional significance of this peculiar localization is not yet known, but we note that several mitotic spindle proteins with a purported role during mitosis were reported to be localized in the postmitotic bridge. Examples include the motor proteins CENP-E (Yen et al., 1991) and MKLP-1 (Nislow et al., 1992), and the inner centromere proteins (INCENPs; Mackay et al., 1993). It is possible that material in postmitotic bridges may be discarded along with the midbody after each cell division, which might explain the reduced levels of Plk1 in G₁ phase cells.

The subcellular localization and cell cycle-dependent pattern of expression of Plk1 are consistent with a mitotic function of this kinase, reminiscent of its putative homologues in *Drosophila* and *S. cerevisiae*. Very little is known about regulatory events occurring during the late stages of mitosis. However, it is clear that the mitotic spindle is the point of action of a major checkpoint controlling the passage through the cell cycle (Hoyt et al., 1991; Li and Murray, 1991), and mitosis is delayed if spindle function is impaired or perturbed (for reviews see Hartwell and Weinert, 1989; Murray, 1992). The phenotypes caused by mutations in *polo* and *CDC5*, respectively, raise the possibility that polo-like kinases may be part of a regulatory pathway acting upon the mitotic spindle. The identification of human and murine Plk1 sets the stage for exploring this intriguing possibility in vertebrate cells.

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